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Positively Frequency-Dependent Interference Competition Maintains Diversity and Pervades a Natural Population of Cooperative Microbes

Highlights

- Positively frequency-dependent interference competition is common in *M. xanthus*
- Positively frequency-dependent selection maintains microbial diversity
- Interference competition appears to be contact dependent and is often lethal

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In Brief

Rendueles et al. show that bacterial strains that are severely inferior competitors at intermediate frequency are competitively dominant at high frequency. Such positive frequency dependence pervades a natural population of *Myxococcus xanthus*, promotes diversity in patchily distributed populations, and is mediated by contact-dependent mechanisms.

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Positively Frequency-Dependent Interference Competition Maintains Diversity and Pervades a Natural Population of Cooperative Microbes

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SUMMARY

Positively frequency-dependent selection is predicted from theory to promote diversity in patchily structured populations and communities, but empirical support for this prediction has been lacking. Here, we investigate frequency-dependent selection among isolates from a local natural population of the highly social bacterium *Myxococcus xanthus*. Upon starvation, closely related cells of *M. xanthus* cooperate to construct multicellular fruiting bodies, yet recently diverged genotypes co-residing in a local soil population often antagonize one another during fruiting-body development in mixed groups. In the experiments reported here, both fitness per se and strong forms of interference competition exhibit pervasive and strong positive frequency dependence (PFD) among many isolates from a centimeter-scale soil population of *M. xanthus*. All strains that compete poorly at intermediate frequency are shown to be competitively dominant at high frequency in most genotype pairings during both growth and development, and strongly so. Interference competition is often lethal and appears to be contact dependent rather than mediated by diffusible compounds. Finally, we experimentally demonstrate that positively frequency-dependent selection maintains diversity when genotype frequencies vary patchily in structured populations. These results suggest that PFD contributes to the high levels of local diversity found among *M. xanthus* social groups in natural soil populations by reinforcing social barriers to cross-territory invasion and thereby also promotes high within-group relatedness. More broadly, our results suggest that potential roles of PFD in maintaining patchily distributed diversity should be investigated more extensively in other species.

INTRODUCTION

Recently developed sequencing technologies have revealed previously unfathomed degrees of microbial diversity in all habitable environments, including soil [1, 2]. Ecological factors expected to promote diversity [3, 4] include spatial structure per se [5] and variable abiotic [4, 6, 7] and biotic [4, 8] environmental features, but the actual contributions of these factors to natural microbial diversity often remain obscure. Among biotic factors, cooperative and competitive social interactions can each affect levels of intra-specific diversity, as can both resource and interference competition [4, 7, 9–11].

Many competitive interactions are frequency dependent, either negatively or positively [12–15]. Negative frequency dependence (NFD) of fitness has long been recognized to promote diversity when the fitness ranks of competitors reverse at a threshold frequency and rarity confers a fitness advantage [14, 16–19]. Fitness-rank-reversing NFD has been documented in many organisms [20, 21], including experimentally evolved populations of bacteria [7, 19, 22]. In contrast, positive frequency dependence (PFD) was predicted by early models to reduce diversity due to the systematic loss of rare types [23–26]. This prediction was supported by experiments with bacteria that engage in toxin-mediated interference competition. When cells of *Escherichia coli* that produce an anti-competitor toxin were mixed with non-producers, producers either increased toward fixation or decreased toward extinction, contingent on their starting frequency and the competition environment [27]. However, more recently developed theory has predicted that PFD should actually maintain diversity, rather than reduce it, when populations patchily occupy spatially structured habitats [18, 28]. In this view, spatial variation in competitor frequencies generates local priority effects such that different genotypes (or species, in community scenarios) numerically—and therefore also competitively—dominate distinct local territory patches.

Interference competition is common among microbes [29, 30] and evolves readily in experimental populations [31]. It is thus likely to play a major role in shaping microbial diversity [9, 11, 32]. However, the prevalence of interference competition among conspecific neighbors in local natural populations, the effect of frequency on such interference-competition fitness relationships, and the implications of any such frequency effects in the maintenance of microbial diversity remain little explored.

Myxococcus xanthus is a soil bacterium that engages in several cooperative behaviors, including motility [33, 34], predation [35, 36], and starvation-induced development into multicellular fruiting bodies [37]. *M. xanthus* soil populations are highly diverse, both genotypically [38] and phenotypically [34, 35, 39, 40], even at very small spatial scales. For example, in one centimeter-scale soil population of *M. xanthus* in Tübingen, Germany, dozens of socially distinct genotypes were found to coexist among only 78 independent isolates [41]. Social interactions

during codevelopment when *M. xanthus* natural isolates are forcibly mixed are often severely antagonistic, even among very closely related strains [42, 43].

Here we analyze the frequency dependence and modes of competitive interactions among isolates from the Tübingen population [41, 43]. All of the examined isolates produce large and similar numbers of spores during starvation-induced development in pure culture [43]. In contrast, in many pairwise 1:1 mixes among these strains, codevelopmental fitness is highly asymmetric, with inferior competitors suffering large reductions in spore production due to the presence of the superior competitor. Pairwise codevelopment fitness ranks among nine isolates were previously found to be strongly hierarchical, thus highlighting the question of how so many genotypes can coexist in a local population [43, 44].

Using these same Tübingen isolates, we tested whether relative fitness between antagonistic strains is frequency dependent during both vegetative growth and codevelopment. We further performed experiments to demonstrate how PFD can maintain diversity in patchy environments. Finally, we tested whether observed antagonisms can be explained by diffusible secretions or rather involve contact-dependent mechanisms.

RESULTS

Rarity Is Strongly Disadvantageous Even for Strains that Are Competitively Dominant at Intermediate Frequencies

We tested the relationship between frequency and fitness in pairwise competition mixes among three focal isolates (A23, A47, and A96) during both vegetative growth on high-nutrient agar (without swarm expansion; see Supplemental Experimental Procedures) and starvation-induced development. For both types of competition, we employed a relative fitness parameter, W_{ij}, that was originally formulated for codevelopment competitions (see Supplemental Experimental Procedures) [42, 43]. Positive values of W_{ij} indicate that a focal strain *i* has higher relative fitness than its competitor *j*, and negative values indicate the opposite. For all three possible pairs, the competitor that was previously shown to be inferior in 1:1 developmental competitions (hereafter always referred to as the $IC_{0.5}$ competitor) [42, 43] was allowed to start competitions in the majority (9:1 and 99:1), as well as at a 1:1 ratio.

At 1:1 mixing ratios, fitness ranks conformed to previous developmental competitions, with A47 winning over both other strains and A96 winning over A23 (Figure 1A; Table S1) [42, 43]. During both growth and development, relative fitness of the $IC_{0.5}$ competitor exhibited extremely strong PFD. Indeed, PFD was so strong that the fitness ranks of the paired competitors reversed strikingly as a function of frequency in all competitions (Figure 1A; Table S1). Thus, among these isolates, strains that are rare within groups are unable to invade common strains during either growth or development, even when the rare strain has a strong fitness advantage at intermediate frequencies.

We also tested for strain-interaction effects on the absolute performance of each competitor in mixed groups by calculating the parameter $C_i(j)$, which quantifies the effect of mixing two strains *i* and *j* on the viable population size of strain *i*, relative to pure culture controls [42]. Negative values of $C_i(j)$ indicate that strain *i* had lower spore production during development (or population yield during growth) in the presence of *j* than in pure culture, and positive values indicate the opposite. In all cases, competitive dominance by the majority strain (positive W_{ij}) was caused solely or predominantly by strongly negative effects of mixing on the performance of the rare competitor, rather than by positive responses to mixing by the majority competitor (Figures 1B and 1C). In other words, during both growth and development, the absolute performance of the rare strain was always much worse in the presence of the majority competitor than in pure culture (usually by multiple orders of magnitude). This pattern indicates the presence of antagonism mechanisms that operate as a function of majority status, irrespective of which strain dominates when strain frequencies are similar.

PFD Is Widespread in a Natural Centimeter-Scale Soil Population

We also analyzed eleven other pairings among the nine Tübingen centimeter-scale isolates that exhibited large fitness asymmetries in a previous study [43] for PFD of both relative fitness (as reflected by W_{ij}) and antagonistic interactions (as reflected by negative $C_i(j)$ values) during codevelopment. On average across all 14 developmental competition pairs (including pairings among A23, A47, and A96), $IC_{0.5}$ competitors that (by definition) lose at intermediate frequency (average $W_{ij} = -4.45$ in 1:1 mixes) win strongly when they start at high frequency (average $W_{ij} = 0.71$ and 2.54 in 9:1 and 99:1 mixes, respectively; Figure 2A). Among individual competition pairs, fitness rank reversed as a function of frequency in all but one case, with the exact shape of the frequency-fitness relationship varying across strains (Figure S1A; Table S1).

Correspondingly, the average sporulation efficiency of $IC_{0.5}$ partners was greatly reduced by their paired competitors in the 1:1 mixes, but not when the former started at high frequencies (Figure 2B; $IC_{0.5}$ mean $C_i(j)$ values of −3.73, −1.02, and 0.06 in 1:1, 9:1, and 99:1 mixes, respectively). Inversely, the absolute performance of strains that win at intermediate frequency was unaffected by $IC_{0.5}$ strains in the 1:1 mixes (mean $C_i(j) = 0.10$) but was strongly reduced by the presence of $IC_{0.5}$ competitors when the latter started at high frequencies (mean $C_i(j)$ values of −1.9 and −2.61 in 9:1 and 99:1 mixes, respectively; Figure 2B). Although the average overall relationship between frequency and unidirectional mixing effects is extremely strong and clear (Figure 2), the frequency interval across which the directionality of antagonism reversed varied across competition pairs (Table S1; Figure S1). In most mixes, significant antagonistic interactions occurred primarily in one direction, as chimerism in these 14 pairwise mixes significantly reduced total group productivity only at the 9:1 mixing ratio (on average across all 14 pairs), and in that case only to a relatively small degree (Table S2).

Strong PFD antagonisms are specifically caused by interactions between distinct natural isolates and are not due to either marker or density effects on the ability of each to strain to develop or compete. In competition experiments between differentially marked variants of the same natural isolate, no significantly negative effects of mixing on spore production were found for any strain at any frequency (Figure S2). Additionally, although total cell density was constant across all sporulation assays, it is also clear that antagonism patterns cannot be explained by

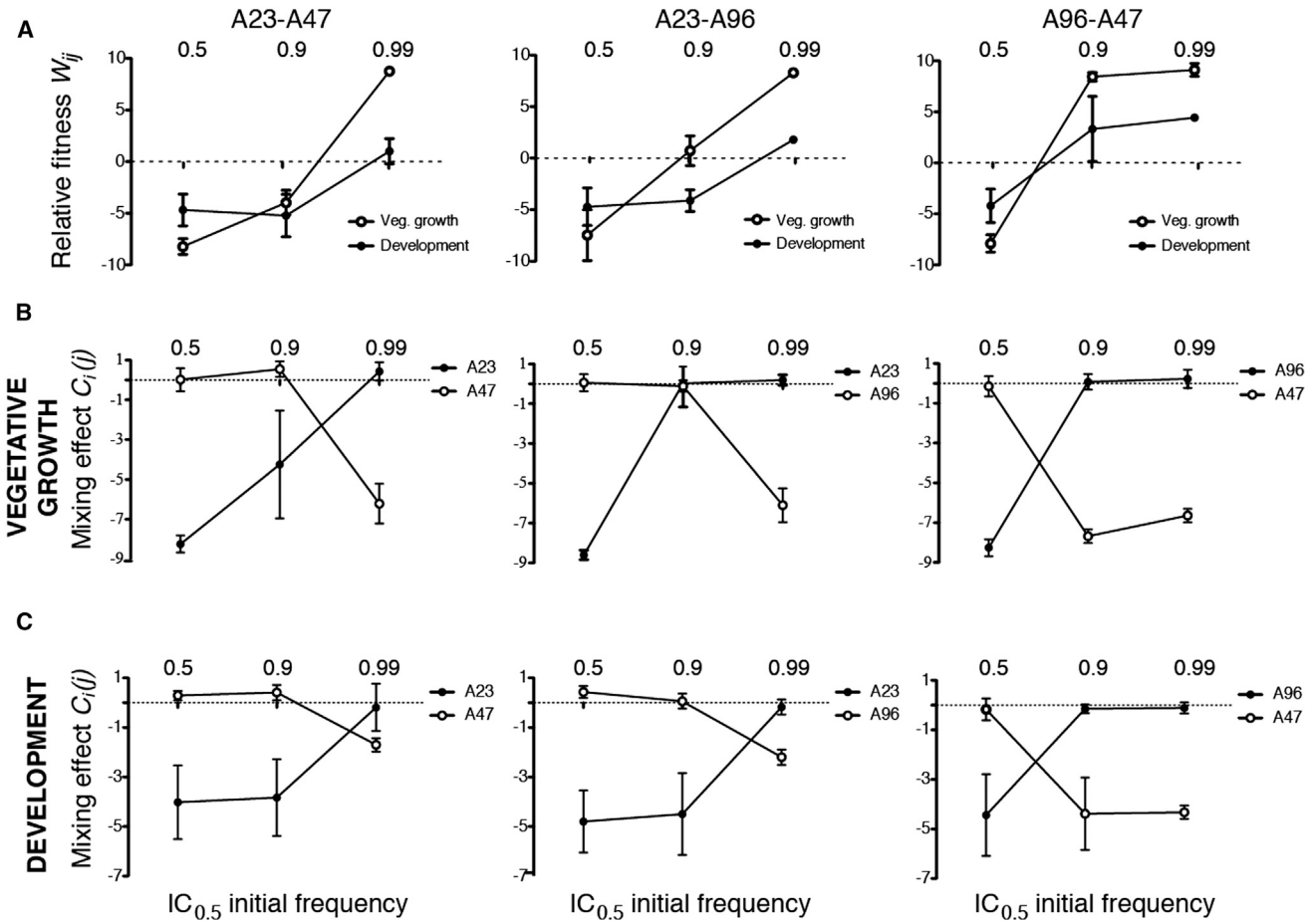

Figure 1. Fitness and Inter-strain Antagonisms among Three Natural Isolates Are Highly Frequency Dependent during both Growth and Development

(A) Relative fitness values (W_{ij}) of the competitor that is inferior in 1:1 mixes ($IC_{0.5}$) during vegetative growth (white circles) and codevelopment (black circles). (B and C) The effects of mixing two strains on the $\log_{10}$-transformed population size of each after co-culture during growth on an agar surface (B) and co-development (C) are given as $C_i(j)$, with i representing the focal strain of interest. Black and white circles represent the inferior and superior competitors, respectively, in 1:1 mixes. Maximally negative $C_i(j)$ values are ~ -8.5 for vegetative growth and ~ -7.0 for codevelopment.
Error bars represent 95% confidence intervals.

genotype-specific density effects. If mere genotype-specific density were responsible for spore production patterns, any one genotype should show the same pattern across mixing frequencies regardless of which competitor it is paired with, but this is clearly not the case. For example, frequency-dependent patterns of spore production exhibited by A47 (Figure 1C, left and right panels; Figure S1G), A96 (Figure 1C, middle graph; Figures S1I–S1K), and A75 (Figures S1B, S1C, and S1E) differ greatly as a function of competitor genotype.

Positive Frequency Dependence Maintains Diversity in Patchy Environments

Theory predicts that PFD can maintain diversity in patchily distributed populations and communities due to local priority effects [18, 28]. We tested this prediction experimentally by performing developmental competitions with two pairs of natural *M. xanthus* isolates (A30 versus A96 and A96 versus A47) in which overall initial competitor frequency was invariantly 0.5 across each of two treatments, but in which the spatial distribution of competitor frequencies was homogeneous in one treatment and heterogeneous in another, with local majority status varying across patchy subpopulations (Figure 3). More specifically, in the spatially homogeneous treatments, each competition arena consisted of four subpopulation patches across which the two competitors were evenly distributed at a 1:1 ratio. In the spatially heterogeneous treatments, each subpopulation patch was initiated with one competitor in a 99:1 majority, with the majority competitor alternating patch-wise to give an overall 1:1 competitor ratio across all four patches. Subpopulation patches were placed in close proximity on a common agar plate so that patches could migrate toward one another and, in the absence of any mechanisms of kin discrimination that operate at the inter-patch level, merge. Arena populations were allowed to compete over two complete cycles of development and one growth phase between the developmental cycles (see Supplemental Experimental Procedures). To maintain population spatial structure that was not eliminated by motility-driven inter-patch migration, patch subpopulations were harvested

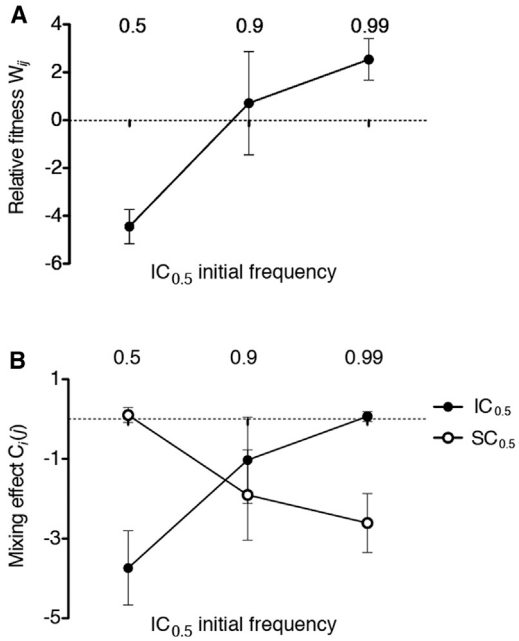

Figure 2. Average Fitness and Mixing Effects across 14 Natural Isolate Pairs during Developmental Competitions Are Highly Frequency Dependent

(A) Average fitness values of the $IC_{0.5}$ competitors across all 14 competition pairings at three initial $IC_{0.5}$ frequencies.

(B) Average mixing effects ($C_i(j)$) on spore production across all 14 competition pairings when inoculated at three initial $IC_{0.5}$ frequencies. Values for competitors that are inferior ($IC_{0.5}$) and superior ($SC_{0.5}$) at a 1:1 mixing ratio are represented with black and white circles, respectively.

Error bars represent 95% confidence intervals.

separately at the end of the first round of development and cultured separately during the growth phase before being placed in the same relative configuration on a common plate for the second round of development.

During both developmental cycles, patches in the spatially homogeneous treatment merged until patch boundaries were not visible. In contrast, in the spatially heterogeneous treatment, inter-patch demarcation lines were clearly evident, indicating the operation of kin discriminatory social incompatibilities between the distinct majority genotypes in neighboring patches (data not shown) and suggesting that such pre-existing social barriers reduced migration across patches.

We used the Shannon index, which is determined by both genotype richness (here maximally two) and genotype frequencies [45], as our measure of diversity. After two developmental cycles, diversity was entirely or nearly eliminated in all replicates of the spatially homogeneous treatment for both competition pairs (Figure 3), with the $IC_{0.5}$ competitor driven to complete or near extinction in all cases. In arenas in which the $IC_{0.5}$ was still present, it was reduced to extremely low numbers (fewer than 350 cells from initial populations of 10^9) and thus should have gone extinct had the competitions been continued through a third round.

In striking contrast, not only were both competitors maintained in all replicates in the spatially heterogeneous treatment, but they were maintained at nearly equal frequencies in all arenas (Figure 3). As expected from the outcomes of non-patchy competition arenas (Figures 1 and 2), diversity was maintained almost exclusively at the inter-patch level in this treatment, as the minority strain went completely or nearly extinct within all patch subpopulations (30/48 and 18/48 patches, respectively), with each competitor dominating the two patches in which it started in the majority. Thus, although inter-patch migration was allowed, it either did not occur at all due to pre-existing social barriers or was insufficient to allow competitors that win at intermediate frequencies to significantly reduce $IC_{0.5}$ frequencies in patches in which the latter started in the majority. In conclusion, PFD maintained biological diversity at nearly maximal levels under conditions in which it would be rapidly eliminated in the absence of PFD.

Diffusible Toxins Were Not Detected

Frequency-dependent fitness-rank reversals cannot be explained by intrinsic differences between competitors that are expressed in pure culture but rather imply strong interactions between strains. We investigated whether the antagonistic interactions documented here are mediated by diffusible molecules or rather are contact dependent, first by asking whether conditioned supernatant from vegetative cultures of each strain affected the growth rate of other isolates for seven strain pairs. No significant effects were found across or within pairs (Figure S3). Because development triggers massive changes in gene expression [37], we also tested whether diffusible secretions might be produced specifically during development. To do so, we assessed the ability of three focal isolates that show similar spore production in pure culture (A23, A47, and A96; one-way ANOVA test for variation between strains, $p = 0.806$) to develop in the presence of liquid buffer that had been preconditioned by each of the other isolates. First, we prepared cell-free supernatant from development cultures submerged in a liquid buffer (MC7). We then qualitatively assessed fruiting-body formation and quantitatively estimated spore production of each strain in presence of MC7 conditioned by each isolate and fresh, unconditioned buffer (see Supplemental Experimental Procedures).

After 5 days, cultures supplemented with pre-conditioned supernatant from any source strain exhibited greater developmental maturity (darker and larger fruiting bodies) than did controls treated with fresh MC7 (Figure 4). Interestingly, development by A23 and A47 in media that had been pre-conditioned by any strain yielded significantly more spores than did development in unconditioned MC7 alone (Figure 4; two-way ANOVA, $F = 13.82$, $p < 0.0001$). This result demonstrates the presence of a diffusible cue to which both A23 and A47 respond that appears to be produced similarly by all three strains. Importantly, no differences were observed between the effects of different pre-conditioned supernatants from the three source strains on either fruiting-body formation (qualitative visual assessment) or spore production for any of the three recipient strains (Figure 4). This result strongly suggests that the severe developmental antagonisms between these strains are not caused by diffusible antagonistic compounds secreted during development.

In theory, a diffusible secretion might hinder developmental performance by an effect other than direct toxicity, such as

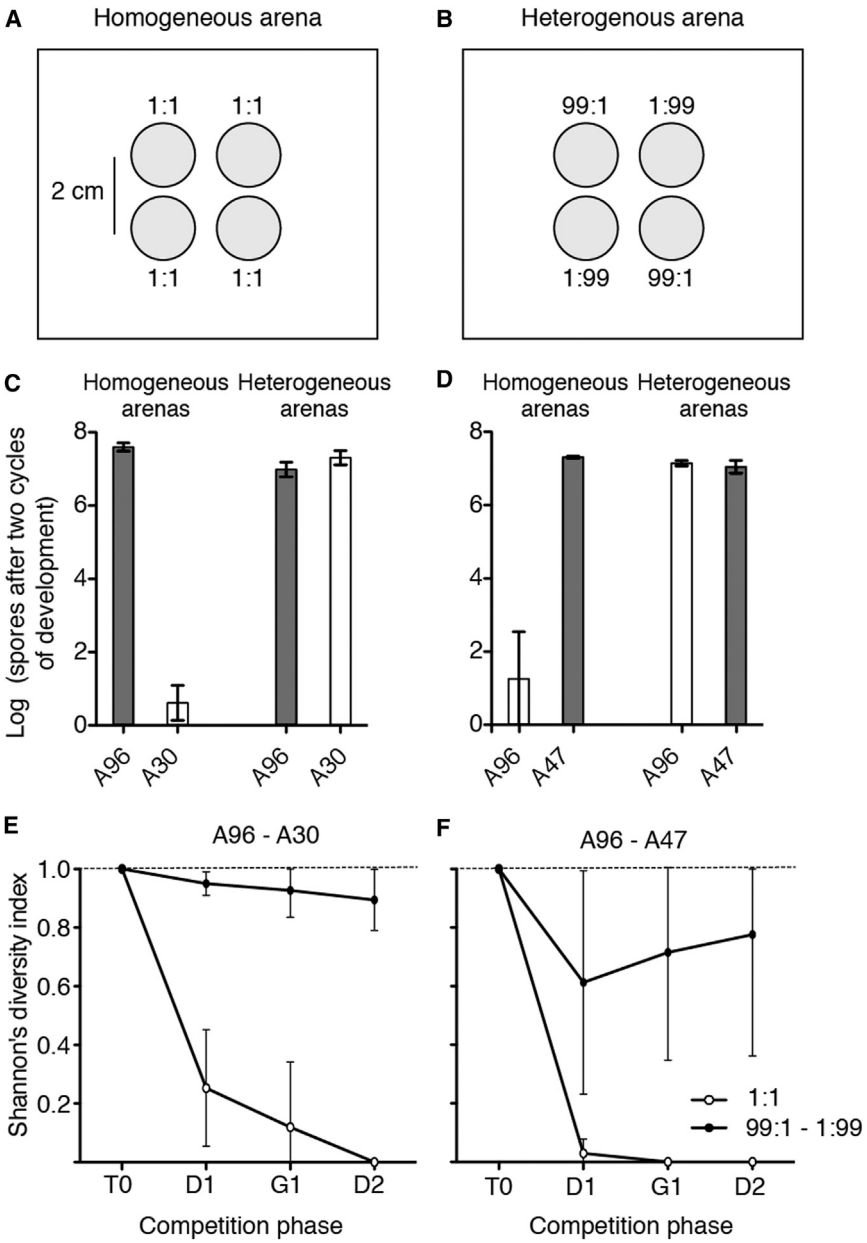

Figure 3. Positive Frequency Dependence Maintains Diversity in Patchily Distributed Populations

(A and B) Each competition arena consisted of four subpopulation patches across which the overall initial competitor frequency in each arena was invariantly 0.5. In homogenous arenas (A), the two competitors were evenly distributed at a 1:1 ratio in all four patches. In the spatially heterogeneous arenas (B), each subpopulation patch was initiated with one competitor in a 99:1 majority. Subpopulation patches were placed 2 cm apart from each other so that patches could migrate toward one another and, in the absence of any mechanisms of kin discrimination that operate at the inter-patch level, merge.

(C and D) Total spore counts of each natural isolate after two cycles of competition for two pairwise competitions, A96-A30 (C) and A96-A47 (D). For each pairing, the $IC_{0.5}$ is represented with white bars. Error bars represent 95% confidence intervals.

(E and F) In competition treatments in which competitors were distributed homogeneously across subpopulation patches, the $IC_{0.5}$ competitor (white circles) is driven to extinction in both competition pairs A30-A96 (E) and A47-A96 (F), whereas both competitors are maintained at high levels when initial competitor frequencies vary across subpopulation patches (black circles). Dashed lines represent the maximum diversity level possible in this experimental system (both strains present at equal frequency). T0: beginning of competition; D1 and D2: first and second rounds of codevelopment; G1: intermediate liquid growth phase.

hindrance of cell-cell adhesion necessary for effective development [37]. Using the same three focal strains, we tested whether a diffusible factor secreted during vegetative growth alters the ability of competitor cells to adhere, but no such effect was found (Figure S3). Taken together, our results indicate that developmental antagonisms are not caused by obligately produced diffusible secretions.

Antagonism during Vegetative Growth Is Often Lethal and Appears to Be Contact Dependent

If antagonisms are contact dependent, we predicted that they would be more pronounced in populations growing on solid surfaces than in populations dispersed in liquid due to lower density in the latter. We thus compared antagonistic mixing effects on strain performance among pairings between A23, A47, and A96 across three environments: nutrient-rich liquid, nutrient-rich agar plates, and during development at 1:1 mixing ratios (Table 1).

Within each competitor pairing, the same strain was antagonized in all three environments (Table 1), suggesting that similar or identical mechanisms of antagonism may operate across environments. In the liquid competitions, the winning strain in all three pairings significantly reduced cell counts of the inferior competitor by more than a factor of ten relative to expectations from pure culture growth (i.e., $C_i(j)$ was always lower than −1.0; Table 1). Even more strikingly, in two competitions (A23-A47 and A96-A47) these antagonisms caused significant decreases in the absolute size of the $IC_{0.5}$ subpopulation (73% and 69% for A23 and A96, respectively; $p < 0.05$ for differences between initial and final subpopulation sizes, one-sample t test), indicating that antagonisms were lethal to many cells even in liquid.

Although antagonisms during liquid growth were large in their population-level effects, such effects were much greater in competitions on high-nutrient agar surfaces (Table 1). In this environment, the vast majority of cells of victimized competitors were clearly killed by the winning strain. This was evidenced by lack

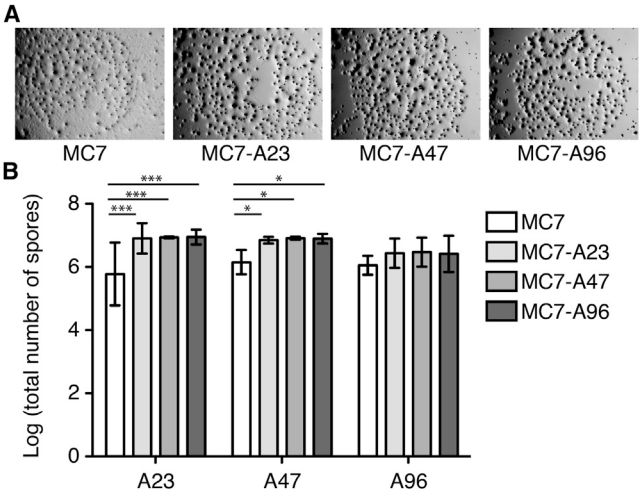

Figure 4. Diffusible Secretions Enhance Rather Than Inhibit Sporulation across Antagonistic Strains

(A) Cultures of A23 were allowed to develop while submerged in MC7 starvation buffer that was either unconditioned (MC7) or previously conditioned by A23 (self-self control, MC7-A23), A47 (MC7-A47), or A96 (MC7-A96). Pictures were taken after 5 days of development (magnification ~ 50×).

(B) Average total spore production by three focal strains after development while submerged in either fresh MC7 media (white bars) or in MC7 preconditioned by each of the three strains (gray bars). *$p < 0.05$; ***$p < 0.001$, post hoc tests with Bonferroni correction for differences from fresh MC7 treatment. Error bars represent 95% confidence intervals.

Table 1. Antagonisms Have Smaller Population-Level Effects in Liquid Than on Solid Surfaces

Strain Pair	$C_i(j)$ Development	$C_i(j)$ Liquid Growth	$C_i(j)$ Surface Growth	p Values Liquid versus Surface
A23-A47	−4.02 ± 1.74	−2.32 ± 1.01	−8.24 ± 0.43	<0.001
A23-A96	−4.79 ± 1.46	−1.65 ± 0.74	−8.60 ± 0.24	<0.001
A47-A96	−4.44 ± 1.55	−1.68 ± 0.67	−8.26 ± 0.43	<0.001
$C_i(j)$ max	~ −7	~ −7	~ −8.5	

$C_i(j)$ values for inferior competitors in pairwise competitions between the three focal strains in three different environments (development, liquid, and surface growth) are shown. Data represent mean ± 95% confidence intervals. The inferior competitor (i.e., the strain with a negative W_{ij}) in 1:1 mixes ($IC_{0.5}$) is underlined. p values are based on post hoc Bonferroni testing. Theoretically maximally negative $C_i(j)$ values are also shown.

of regrowth of the antagonized strains from post-competition cultures on selective plates containing antibiotic to which only the winning strain is sensitive (Figure S4). In most replicates across all three pairings for all three frequencies tested, the entire losing subpopulation was completely killed (38/56), and in the remaining 18 cases it was greatly reduced. Overall, the absence of negative supernatant effects combined with increased degrees of cell death on surfaces relative to liquid cultures strongly suggests that antagonisms are contact dependent.

We also conducted a fourth type of competition experiment in which 1:1 competition cultures were allowed to swarm outward on high-nutrient agar surfaces. After one week of swarming on both hard and soft CTT agar, samples from four locations evenly distributed around each swarm perimeter were transferred onto CTT hard agar selecting for each of two competing isolates. Although the swarming rates of these three strains are indistinguishable in pure culture ([34] and unpublished data), in the mixed competitions the inferior competitor was completely absent from the swarming edge in all replicates. Thus, inclusion of population-level swarming as a component of fitness allowed antagonisms to even more severely eliminate competitors.

DISCUSSION

In concordance with recently developed theory [18, 28], we have experimentally demonstrated that PFD of fitness can maintain, rather than purge, diversity in patchily structured populations in which the majority genotype varies across local patches. Although PFD has been documented in other microbial systems, the role of PFD in maintaining natural microbial diversity, whether at the population or community level, remains largely unexplored. The pervasiveness of extremely strong PFD in a centimeter-scale population of *M. xanthus* documented here suggests that similar patterns may be found among other microbial species. More broadly, PFD also occurs among plants [13] and animals [46], including in human economic systems [47]. Our empirical results suggest that the potential for PFD to help maintain biological, cultural, and technological diversity in spatially structured systems of all types should be more broadly investigated.

PFD traits are expected to generate self-organized population structure in which distinct areas differ in their most common genotype [28]. PFD reinforces genotype-territory boundaries by disfavoring migrants that are locally rare after crossing such boundaries. In our experiments, we showed that patches in which $IC_{0.5}$ competitors were at high initial frequency (0.99) were not overrun from neighboring patches by the alternative competitor (that dominates in 1:1 competitions), thus allowing diversity to be maintained across patches. Moreover, PFD-mediated diversity is expected to increase with the degree to which populations are spatially fragmented by uninhabited patches [28].

Myxococcus cells live in patchily distributed kin groups in the soil [41, 44]. Groups that form fruiting bodies on soil particles often include detectable genetic diversity, but that diversity is usually of endemic origin and tends to be low relative to diversity across kin groups at millimeter and centimeter scales (S. Kraemer, S. Wielgoss, and G.J.V., unpublished data). Laboratory experiments suggest that kin discriminatory traits often prevent genetically distinct neighboring kin groups from freely merging upon encounter in the soil. Such kin discrimination during heterotypic colony encounters is prevalent among the centimeter-scale isolates examined here [43] and reduces co-aggregation into chimeric fruiting bodies at the interface of oncoming colonies formed by the three focal strains A23, A47, and A96 (unpublished data). These colony-merger incompatibilities alone are predicted to increase kin-group structure in soil populations (above that expected from simply clonal reproduction on a structured surface by motile cells [43, 48]). Indeed, in our patchy competition experiments, clearly visible social barriers were established between neighboring patches dominated by different strains that reduced or entirely prevented patch merger, whereas such merger occurred readily when patches were homogenous with respect to initial competitor frequency.

The severe PFD among centimeter-scale neighbors documented here should reinforce fragmented kin-group structure and local diversity generated by colony-merger incompatibilities. If some such incompatibilities were to only reduce, but not entirely prevent, cell-cell encounters between members of distinct kin groups, PFD interference competition should nonetheless reduce the frequency of successful cross-patch invasion events. Cells that migrate into territory numerically dominated by a distinct social type will suffer the PFD disadvantage of being rare. Together, then, as shown in Figure 3, colony-merger incompatibilities [43, 49, 50] and PFD acting against inter-group migration can promote the maintenance of structured diversity by benefiting genotypes that would be severely inferior if local migration were unlimited and genotype frequencies were spatially homogenized. Also, although all of the strains examined here are highly proficient at development in clonal groups [39, 43], PFD antagonisms may also function together with colony-merger incompatibilities to limit the spread of socially defective cheaters across kin groups and thus increase equilibrium levels of within-group cooperation [51].

Of course, our results do not explain how, if many genotypes have an advantage only at high frequency, those genotypes became established in local territory patches in the first place. In one scenario, a new mutant might arise at or near the edge of a patch subpopulation and migrate to occupy an unoccupied patch. There appear to be many soil micro-patches unoccupied by *M. xanthus* at any given time, as a substantial fraction of soil samples do not yield fruiting bodies, even from areas in which *M. xanthus* is common ([41] and unpublished data). Alternatively, patch subpopulations might first diverge in evolutionary isolation at traits that affect developmental competitiveness before subsequently encountering one another due to migration.

The role of PFD in interference competition on structured surfaces has been previously examined for unidirectional antagonisms mediated by obligately produced diffusible toxins [27, 52]. Working with toxin-producing bacteria that exhibit PFD, Chao and Levin showed that toxin production remained beneficial even at very low producer frequencies [27]. In contrast, working with yeast, Greig and Travisano found that the cost of toxin production can outweigh benefits derived from harm to competitors when both overall cell density and toxin-producer frequency are sufficiently low [52]. Indeed, not only was an outright advantage to toxin production lost when both total density and producer frequency were very low, but producers were at a slight disadvantage (a few percent). This result showed that fitness ranks in a PFD relationship can reverse in a frequency-dependent manner, at least when overall population density is sufficiently low.

Our results differ greatly from those of Greig and Travisano. First and foremost, the *M. xanthus* antagonisms documented here are, in all but one case, bidirectional between competitors. In Greig and Travisano's yeast experiments, toxin non-producers exhibited only a slight advantage over producers when the latter were at very low density and frequency. Moreover, this advantage by non-producers was due to resource competition (differential metabolic costs) rather than interference competition [52]. In our experiments with bacteria, fitness-rank reversals were caused by bidirectional interference competition (Figures 2A and S1A). In almost every competition pair, the strain that strongly antagonized its partner at a 1:1 mixing ratio itself became a victim of strong antagonism when its frequency was reduced. Second, fitness-rank reversals in *M. xanthus* competitions do not require either extreme frequency fluctuations—reversal thresholds were at $IC_{0.5}$ frequencies between either 0.5 and 0.9 (nine cases; Figures S1D, S1F–S1L, 2B, and 2C) or between 0.9 and 0.99 (four cases; Figures 2B, S1B, S1C, and S1E)—or low population densities.

In our experiments, antagonistic interactions during both development and growth were quantitatively severe and in many cases caused outright extinction of the losing subpopulation. Multiple mechanisms of lethal contact-dependent interference competition by bacteria have been reported, including those mediated by type VI secretion systems [53], *rhs*-like proteins [54], and *cdi*-like contact-dependent inhibition systems [55]. The *M. xanthus* genome carries homologs to several such systems (*MXAN*_4800–*MXAN*_4815, *MXAN*_5799, *MXAN*_6679), some of which are involved in motility and development [56, 57]. Future work is required to address whether any of these homologs are involved in the pervasive antagonistic interactions reported here, whether the molecular mechanisms causing PFD are functionally linked to mechanisms causing colony-merger incompatibilities [43], and the evolutionary mechanisms by which PFD interference competition first evolved [58].

A novel instance of PFD was previously shown to evolve rapidly and de novo in an experimental laboratory population of *M. xanthus*. Through the effect of a single mutation, a socially defective cheater that exhibited a negatively frequency-dependent fitness relationship toward an ancestral cooperator [59, 60] was transformed into a derived, socially proficient cooperator that exhibited positively frequency-dependent fitness dominance over the ancestor [59, 60]. This reversal of NFD into PFD by one mutation suggests that new forms of PFD may evolve easily in natural populations and thereby increase PFD-mediated diversity.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.04.057.

AUTHOR CONTRIBUTIONS

O.R. and G.J.V. designed the experiments, analyzed the data, and wrote the manuscript. O.R. and M.A. performed the experiments.

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